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NEOPLASTIC DISEASE

Assessment of Lymphoid Molecular Clonality in Canine Thymoma

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Summary

The aim of this study was to document the molecular clonality of lymphoid cells in canine thymoma using polymerase chain reaction for antigen receptor rearrangement (PARR). Fifteen formalin-fixed and paraffin wax-embedded samples of canine thymoma were analyzed for T- and B-cell receptor clonality. Two of these 15 cases were excluded from the study due to insufficient DNA concentration. Twelve of the 13 remaining samples (92.3%) showed a polyclonal lymphoid component and in one case the lymphoid component was monoclonal (T-cell clonality). PARR could therefore be a useful tool for differentiating canine thymoma from canine mediastinal lymphoma.

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Thymoma and lymphoma are the most common causes of mediastinal masses in dogs (Yoon *et al.*, 2004). Thymoma is a neoplasm originating from the epithelial cells of the thymus that are admixed with non-neoplastic lymphoid cells, while lymphoma is characterized by a neoplastic proliferation of lymphoid cells (De Mello Souza, 2013). In dogs, these two tumors have different prognoses and treatment options and being able to differentiate them has a major clinical relevance (De Mello Souza, 2013). Cytological samples from mediastinal masses may be difficult to interpret and the reported cytological accuracy for the diagnosis of mediastinal lesions in dogs varies from 40% to 80% (Atwater *et al.*, 1994; Pintore *et al.*, 2014). Histopathology is considered more reliable for the diagnosis of mediastinal lesions, although a definitive diagnosis may be challenging in cases of thymoma with few epithelial cells.

Polymerase chain reaction for antigen receptor rearrangement (PARR) has recently become widely used for the diagnosis of lymphoma in dogs and cats (Burnett *et al.*, 2003) and may also help in the distinction between mediastinal lymphoma and thymoma. This technique detects and amplifies via polymerase chain reaction (PCR) genes encoding particular chains of the immunoglobulin (for B cells) or T-cell receptor (for T cells) molecules in the lymphoid population of a sample. The resulting PCR products are analyzed by capillary gel electrophoresis. A clonal sample is reported if one or several discrete bands of appropriate DNA size are seen on the gel and distinct fluorescent peaks are seen on an electropheretogram. A polyclonal sample is reported if either no or multiple bands or peaks are seen. Lymphoid cells in lymphoma are typically monoclonal as they are derived from the same precursor cell (Burnett *et al.*, 2003);

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however, there is limited information about the clonality of the lymphoid component of canine thymoma. The aim of the present study was to evaluate the molecular clonality of the lymphoid component in canine thymoma, primarily assessing whether PARR performed on biopsy samples could be used routinely to distinguish this neoplasm from mediastinal lymphoma.

Computerized databases from two referral centers (Animal Health Trust, Newmarket and Dick White Referrals, Six Mile Bottom, UK) were searched for confirmed cases of canine thymoma diagnosed by histopathology. A board-certified veterinary pathologist reviewed all of the samples, with immunohistochemistry for cytokeratin requested as needed (using monoclonal mouse anti-cytokeratin antibody; Dako, Glostrup, Denmark, catalogue number MNF116; dilution 1 in 200). DNA was isolated from sections taken from the paraffin wax-embedded tissue (DNeasy Blood and Tissue Kit®, Qiagen, Manchester, UK) as per the manufacturer's instructions, with minor modifications. The sections were added to a 2 ml tube (Safe-Lock Tube, Eppendorf, Stevenage, UK) and the wax was removed by heating the sections to 70°C for 10 min in 400 µl of ATL buffer (from the extraction kit). The samples were then centrifuged at 11,863 g for 2 min and the solidified wax ring was removed from the top of the ATL using a pipette tip. Proteinase K (40 µl) was added to the tube and the samples were incubated at 56°C with constant shaking (800 rotations per minute) in an incubator (Vortemp 56 Shaking Incubator, Labnet International, Edison, New Jersey, USA) before completion of the tissue extraction protocol. The resulting DNA was eluted in 100 µl of elution buffer before being stored at −20°C prior to analysis. The concentration of DNA was measured (Qubit dsDNA BR Assay Kit, Invitrogen, Paisley, UK). Samples showing insufficient DNA concentration to allow addition of 50 ng of DNA were excluded from the study, as the low quantity of DNA may have decreased the assay sensitivity. Samples with marginal, good and high DNA concentration were included in the study and the DNA concentration was recorded. The presence of amplifiable DNA was confirmed by performing a PCR for the succinate dehydrogenase (SDHA) gene (Peters *et al.*, 2007) and for the presence of PCR inhibitors using an exogenous internal control (internal amplification control [IAC]) assay (Nolan *et al.*, 2006) by real-time PCR as described previously, using a master mix (GoTaq G2 Hot Start Colorless Master Mix, Promega, Southampton, UK) in a specific instrument (Stratagene MX-3005P, Agilent Technologies, Stockport, UK). Samples that failed to have amplifiable DNA with the SDHA assay were excluded from the study. Samples that had a negative canine DNA control (IAC), but for which the quantity of DNA extracted and measured was considered suitable for analysis, were not excluded, as this could have been due to presence of pigments acting as inhibitors. If PCR inhibitors were identified using the IAC assay, 1 µg of bovine serum albumin (BSA) was added to the PCR reaction mixes for the SDHA and IAC assays, and the analysis was repeated. In samples for which inhibition was not evident with reanalysis, an equivalent amount of BSA was added to the PCR reactions for the clonality analysis. PARR for the B-cell immunoglobulin heavy chain locus (IgH) and T-cell receptor gamma (TCRγ) locus was performed using methods described previously (Vernau and Moore, 1999; Burnett *et al.*, 2003; Keller and Moore, 2012).

The resulting products were analyzed by capillary gel electrophoresis using specific software (QIAxcel Advanced System, QIAxcel ScreenGel Software, Version 1.4, Qiagen) and represented as electropheretograms. Results were deemed clonal when there were identical, discrete electrophoretogram peaks, which were a minimum of twice the height of the other amplified products, present in both reaction repeats (Keller and Moore, 2012). As several primers were used, both polyclonal and clonal results were expected to express several peaks. The relative heights of the peaks was therefore the main criteria used to distinguish between clonal and polyclonal samples. Polyclonal controls were obtained from fresh thymic and lymph node tissue obtained *post mortem* from a juvenile dog without evidence of lymphoproliferative disease. The positive control material was obtained from a peripheral blood sample obtained from a leukemic dog that had previously yielded clonal results (Fig. 1).

Fifteen samples were selected for the study, including four tru-cut and 11 intraoperative excisional biopsy samples. Breed, sex, age and type of sample are summarized in Table 1. The age of dogs with thymoma was 8.5 ± 1.5 years (mean ± SD). The represented breeds included Labrador retriever ($n = 4$), golden retriever ($n = 2$), flat coated retriever ($n = 2$), German shepherd dog ($n = 2$), Rhodesian ridgeback ($n = 1$), Lhasa Apso ($n = 1$), Jack Russell terrier ($n = 1$) and crossbred ($n = 2$). After reviewing the samples, the original diagnosis of thymoma was confirmed for all of the 15 samples. Cytokeratin IHC was performed for three samples. DNA was extracted and measured for 15 samples. Two of these samples (both obtained by tru-cut biopsy) showed insufficient DNA concentration and were therefore excluded from the study.

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